THE MOST ABUNDANT GROUPS OF BACTERIA IN SOIL[1]

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The microflora of soil has been under investigation for at least 70 years; and yet there is at the present time no publication which sums up the existing information in a form sufficiently concise to serve as a guide to any one who is beginning research in soil bacteriology or who has not had the opportunity to follow the literature on the subject in its year-by-year development. A beginner in this field is apt to be assigned the task of plating one or two samples of soil; but after the colonies have developed about all he can do with them is to count the numbers—a matter of little significance in soil bacteriology. He may speculate as to what the various organisms are, and which kinds are of importance; but unless he is associated with someone well versed in the field, he has difficulty getting the information in fairly concise form.

The Significance of Soil Flora Studies

Before giving much time to studying this subject, one is naturally interested in the question whether such a study may have any conceivable present or future bearing on soil problems of a practical nature. It must be admitted right at the start that it has no such bearing at the present time. Most of the soil bacteriological methods that have been pursued in the past have been of an entirely different nature; in fact Lochhead with his associates (29, 30) have sometimes seemed to offer about the only assistance to the present author (6, 9, 10, 15) in his advocacy of the soil flora method of approach. Nevertheless, it still seems that, considering how much remains to be learned about the general soil flora, and how few practical lessons to agriculture have been obtained from the other, more intensively pursued, methods of investigation, practical results may some day derive from studies of the sort outlined here. Before taking up the main subject matter of the present paper, however, it seems well to discuss other types of soil bacteriological investigation that have been followed in the past.

Investigations of the nitrogen cycle. The importance of nitrogen transformations and the probable agency of bacteria therein was appreciated in early days of bacteriology; and investigators such as Winogradsky (46), Omelianski (32) and Beijerinck (1) gave much attention to the special groups—nitrifiers, denitrifiers, nitrogen-fixing organisms, and ammonifiers—which take part in such processes. These classic investigations were fundamental, and the information they furnished as to the importance of bacteria in soil is today regarded as elementary. They led to the isolation of the legume nodule bacteria and to the use of these organisms for soil inoculation—which is sometimes regarded as the one significant change in soil practice which has resulted from bacteriology.

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They also explained the reasons for many agricultural practices, such as crop rotation, composting, and the like, which were already in use before bacteria were known; and they have taught us improved methods for composting or green manuring so as to avoid harmful effects from crop residues in soil.

Incidentally, these early investigations revealed so many kinds of organisms unable to grow on ordinary media that the plate count was quite discredited because it probably represented only a small part of the actual soil flora; and there seemed no valid reason for intensive studies of the bacteria that were able to develop on such plates.

The Remy-Löhnis method. A natural outgrowth of this last mentioned consideration was the suggestion of methods depending on other principles than plating. One of the first and most important of these was proposed by Remy (35) in 1902. He made an attempt to determine the physiological functions of a soil by placing weighed amounts (usually 10%) in sterile solutions of known constitution into 1% peptone solution in order to measure ammonification by determining the ammonia produced in a given length of time; and similarly into other solutions favoring respectively the nitrifying, denitrifying and nitrogen-fixing bacteria. Almost simultaneously, Hiltner and Störmer in 1903 (20) proposed a similar method, but inoculated with successive dilutions of soil down to 0.001 mg., and determined the amount of chemical change produced by the greatest dilution which would allow the reaction to occur. These two methods were compared and contrasted in the literature, each vigorously defended by its exponents; but the Hiltner and Störmer method was taken up by few other than its authors, while the Remy method, largely because of its advocacy by Löhnis (31), came into quite wide use. Löhnis modified the method slightly, chiefly by using soil extract as the basis of the solution in which the physiological tests were made. After years of work, principally in Germany and America, it became evident that the results obtained did not parallel actual conditions in the field, and the method gradually fell into disuse. It was illogical, of course, because the biological activities taking place in one of these artificial media were not necessarily the same as those occurring under natural conditions.

About the same time that Remy proposed his method, Withers and Fraps (18, 51) in the United States, developed a similar method. It differed from the Remy procedure in that the ingredients whose decomposition was to be studied were added to a standard soil which was then sterilized and inoculated with the soils under investigation. Logically this was an improvement over the Remy method, as sterile soil presents more natural conditions for the growth of soil bacteria than do solutions. This method was further developed by Stevens and Withers (40) but never came into wide employment. It still presented the theoretical objection that study was being made of a mixed flora, of which entirely different species might predominate under natural conditions; and in actual practice it gave no better correlation with field conditions than did the unmodified Remy method. It was soon dropped, the more willingly, because it presented greater technical difficulties of analysis than did the solution method.

Microscopic methods. In 1917 the writer (7) proposed a method of examining

soil (stained with rose bengal) under the microscope to show bacteria. For certain purposes the method is interesting, but it was never claimed to yield practical results, nor to be of much significance in itself. It is not even a satisfactory means of counting bacteria in soil, as it requires more numerous bacteria than those in ordinary field soil in order to yield a fairly reliable count. It has, however, contributed one point of value: it has shown that plate counts, made under proper conditions, although smaller than total counts of bacteria in soil, are not so far from correct as some soil bacteriologists had predicted.

The method, with slight modification, was taken up by Winogradsky (48, 49, 50), when he resumed soil investigations in the 1920's, and made part of his "direct" method of studying soil bacteria. He drew important conclusions from it, to be discussed in the following pages; but most of his work was with other methods. The whole of his procedure does not seem to have been taken up by anyone else—perhaps because it was complicated, or possibly because his description of the steps was too vague for easy following by others.

A more important modification of the microscopic method was made by Rossi (37) and later strongly advocated by Cholodny (2, 3). This method is to bury a slide (wholly or partially) in soil and to stain the film of microörganisms which becomes attached to its surface after a short incubation; but because its results are hard to put on a quantitative basis, it does not seem likely to be of practical value.

Soil deficiency tests. One of the steps in Winogradsky's "direct" method (50) was to mix soil with mannitol and water into a sort of paste, and to mold it into a plaque, upon which colonies of *Azotobacter* develop spontaneously on incubation. Sometimes these colonies fail to appear, and this was found in many cases to be due to phosphorus deficiency in the soil. Nearly 15 years previously Christensen and Larsen (4) had proposed the growth of Azotobacter as an indicator of deficiency of this element; and Winogradsky seemed to have a simpler method for thus using it. For several years, therefore, the method was extensively investigated, in comparison with chemical tests of soil, plant tests, and growth of other microörganisms (usually fungi) as indicators. It seemed for a time that one or more of these methods might prove useful; but the final outcome of the work has been rather disappointing. None of the methods for employing microorganisms as indicators of soil deficiency has proved promising enough to come into general use.

Present tendencies. But for the stimulus which has been given soil bacteriology by the study of antibiotics (see next paragraph), one might almost characterize its present tendency as being one of defeatism. All the above methods have been tried in the hope of getting practical information for agriculture, but except in one or two instances they have been found wanting. What more is there to do? To judge by the recent decrease in number of papers in soil bacteriology (excluding the subject of antibiotics) the general answer seems to have been: Nothing. Rather than take such a discouraged point of view, however, the present writer prefers to think that we may well get worthwhile results by abandoning short-cut methods, and going back to a laborious pure culture study of the general soil

flora, in the hope of learning the functions of the bacteria, one by one. Certain encouragement for such a procedure is derived by considering recent discoveries in the field of antibiotics.

Studies of antibiosis. Of recent years an entirely new aspect of soil bacteriology has developed which is certainly practical—although not agricultural. The studies of Fleming (17), Dubos (16), Waksman (44), and others have introduced the field of antibiotics. This subject is certainly not soil bacteriology in its strict sense; and yet it is such an important one that it is engaging the attention of more soil bacteriologists than any other field. It is not part of what are here termed soil flora studies; and yet the very fact that organisms of such unexpected practical value as those producing antibiotics have been picked up from soil plates suggests that there may be other organisms able to grow on these plates, which are still unstudied, but which may be of great importance in other unforeseen ways. In other words, the study of antibiosis indicates, as nothing else has yet done, the importance of learning more about the general soil flora. Little enough is yet known along that line, and the present review is presented in the hope of stimulating interest in this neglected field.

Methods of Classification

In beginning a study of the soil flora, the first consideration must be the classification of soil microörganisms into broad general groups. Three methods of general grouping appear to the author to be worth considering here: classification by botanical groups; classification by adaptation to laboratory media; Winogradsky's grouping into zymogenous and autochthonous types. Each of these methods will be discussed in turn.

Classification by botanical groups. This method of grouping seems the most natural to the biologist; yet from the standpoint of soil bacteriology it is not necessarily the most satisfactory. Such a classification is essentially as follows:

Higher fungi
Actinomycetes
Eubacteriales:
    Non-spore-formers
    Spore formers.

In practice such a classification presents certain difficulties. Aside from the fact that no attention is given to function of the organisms, it also has to be recognized that some of the divisions are not clear-cut. The Higher Fungi (molds) and the spore-forming bacteria (*Bacilliaceae*) are easily recognized, but there is no sharp distinction between the non-spore-formers and the Actinomycetes. The typical members of the latter group (*Streptomyces spp.*) are easily enough recognized; but the gradation, through intermediate forms with meager branching if any, into the typical non-spore-former, is so gradual that no sharp line can be definitely recognized.

Classification by adaptation to laboratory media. For practical purposes, the soil bacteriologist often thinks of soil organisms as falling into two groups: (*a*)

those growing on ordinary media (e.g., gelatin, or peptone media); (*b*) those requiring special media, or failing to grow at all under laboratory conditions. Such a classification does not go very far, but it does have certain very distinct advantages. It puts the autotrophic bacteria (like the nitrifiers) into one class, and the general decomposition bacteria (ammonifiers, etc.) into the other; and considering the difference in the methods for studying the two groups, this distinction is of practical value in the laboratory. On the other hand, the distinction is no more clear-cut than that between the Actinomycetes and the non-spore-formers. The term "ordinary media" is far from definite; and it is possible to make various modifications of ordinary peptone formulae which can adapt them to organisms formerly unable to grow under such conditions. Furthermore such a classification leaves one in doubt what to do with bacteria that grow *best* on some special synthetic medium but do grow after a fashion on ordinary peptone agar.

Winogradsky's grouping. Probably one of the most significant ideas that has ever been introduced into soil bacteriology is Winogradsky's (50) grouping of soil microörganisms into "zymogenous" and "autochthonous" types. It is interesting that this Russian bacteriologist, who in his younger days (46) first showed how to grow autotrophic bacteria in the laboratory, in his later years has contributed such an important conception as the distinction here discussed. The distinction in question is best understood by substituting for "autochthonous" the more familiar term "indigenous" or even the still more English word "native". This group of indigenous bacteria may be regarded as always numerous in soil and not fluctuating much in numbers, carrying on activities which require no nutrients or sources of energy other than those normally present in soil. Winogradsky regarded them as primarily small cocci—a point to which the present author (as discussed below) takes exception without, however, questioning the essential validity of Winogradsky's conclusion. The "zymogenous" flora, on the other hand consists of the actively fermenting forms which require for their activity ingredients that are quickly exhausted; hence these organisms may, under proper conditions, increase rapidly to large numbers, and then equally quickly return again to such low numbers as not to be detectable by ordinary analytical methods.

This broad grouping of soil bacteria seems fundamental. It bears no direct relation to ability to grow on laboratory media, and none whatsoever to botanical groups; but that does not detract from its value. Indeed, the very fact that it cuts across the botanical groups makes it possible to use them for further subdivisions, which prove quite useful once the fundamental separation of zymogenous and indigenous types is recognized. Such a classification will be followed here.

The Zymogenous Flora

The zymogenous types, as above mentioned, are those which take part in the rapid fermentative processes, therefore increasing to large numbers whenever furnished with the special nutrients to which they are adapted, and then, after

the process is complete, subsiding to minimal numbers until another occasion for active growth occurs. It can readily be understood that this group includes those bacteria which take part in the transformations of nitrogen as well as most of the other processes by which organic matter is made available to plants. The organic material in question is not normally present in soil, and when added to soil rapidly disappears; it undergoes successive stages of decomposition, and as each stage calls for its own type of microörganisms, it can be seen that its incorporation in soil may stimulate successively various groups of zymogenous species. For practical purposes we can divide this flora into those organisms which require special media for laboratory cultivation and those which grow on ordinary media.

Organisms requiring special media. Prominent in this group are to be mentioned: the nitrifiers, the nitrogen-fixing bacteria, and cellulose decomposing organisms. All three groups, although among the most important microörganisms of soil, occur naturally in such small numbers that they can be obtained in plate culture only after repeated transfers through enrichment media. Methods in use for obtaining them are still essentially those worked out in the early days of soil bacteriology by Winogradsky (46, 47) and Omelianski (30). The nitrifiers are distinctly autotrophic, and do not ordinarily grow on organic media; in fact, it was formerly supposed that they would not live in the presence of organic matter. The nitrogen-fixing and cellulose-decomposing bacteria are not autotrophic, as they require an organic source of energy, and they are not quite so poorly adapted to ordinary media as are the nitrifiers; nevertheless it is ordinarily necessary to use special media for their enrichment and isolation.

Among other bacteria that fall in this group should be mentioned the sulfur-oxidizing organisms, and the acid-fast forms. The latter are specially interesting, as some insist (e.g., H. L. Jensen, 24) that they belong in the genus *Mycobacterium* with the tubercle organism. Their significance in soil seems questionable, since the best known forms seem to be primarily concerned in the decomposition of hydrocarbons (e.g., paraffin), a process which probably is not important in ordinary agricultural soils.

Because of the small numbers in which these organisms requiring special media occur in normal soils, little will be said about them here. The importance of some of their activities is manifest; but their study is undertaken mostly by specialists; and the general student rarely has occasion to try to isolate or to identify them. When such methods are wanted one should consult references cited in text books, as that of Waksman (43), for instance.

Organisms growing on peptone or gelatin media. It can be said that the zymogenous types which grow on ordinary bacteriological media are ammonifying forms, or at least organisms that take part in the various stages of degradation of organic matter, even though not all of them result in its final conversion into ammonia. These organisms may be classified according to botanical groups as follows.

(*a*). *Higher fungi.* The majority of the higher fungi found in soil are commonly referred to by the indefinite, but sometimes convenient, term "molds". They

belong to several families of fungi, and a great variety of species have been identified. Publications describing such forms have appeared in the past; for example, C. N. Jensen (23), Waksman (42, 45) and others (19, 22, 28, 33, 34, 41). These forms do not ordinarily appear on the same plates as those designed to develop the bacterial flora, because of the predilection of molds for rather acid media of high carbohydrate content. It is, however, very easy to prepare media which bring forth numerous colonies of such fungi from almost any soil; the chief difficulty lies in interpreting plate counts in terms of actual mold activity in soil. It is generally assumed that the great majority of the mold colonies come from spores; and it is obvious therefore that a single profusely sporulating fungus, even after passing into an inactive state, might give a higher count than numerous individuals of an actively growing, but non-sporulating fungus. It is hard therefore to get a good idea of the extent of vegetative mycelia in the soil, especially considering that the microscope is of little help. The writer's most commonly employed microscopic method (11) for demonstrating bacteria in soil fails to show molds; and although modifications of the microscopic method (7, 8, 27, 37) have been devised which do show fungi, no one has devised a method for putting such results on a quantitative basis.

Although we lack the data, therefore, for definite quantitative statements, the rôle of fungi in soil seems to be fairly evident. Under ordinary conditions they probably exist primarily in the form of spores; but when supplied with organic matter (especially vegetable) under acid conditions, or conditions where high H-ion concentration can occur during fermentation, the fungi become active. Whether certain species take part in certain stages of this fermentation and others in other stages has not yet been definitely established. Presumably that is the case. In any event, the fungi definitely belong to the zymogenous flora of soil.

(*b*). *Actinomycetes.* Much of what has just been said about the true fungi, can be said for the intermediate group, actinomycetes. The true actinomycetes are filamentous in vegetative form, yet colonies on the plates arise in most cases from spores, not from filaments. The filaments are almost as difficult to demonstrate microscoptically as in the case of higher fungi, although the Rossi-Cholodny (2, 3, 36) method does show them in a semi-quantitative manner. Actinomycetes are presumed to take part in much the same kind of activity as higher fungi, but prefer neutral or weakly basic conditions rather than acid.

The numbers of Actinomyces colonies that develop on plates from soil is surprisingly constant. The writer has plated countless soil samples during the past 40 years, and has rarely found an Actinomyces count (i.e., presumably a spore count) of less than 5 million or more than 30 million per gram. It seems strange, indeed, that a spore count of a zymogenous group of organisms should be so large and so constant; and it is easy to think of the actinomycetes as being, rather, a group of indigenous species. Nevertheless the writer's considered opinion is that such forms, although indigenous to soil in the ordinary sense of the term, are not "autochthonous" in the sense meant by Winogradsky. They are organisms whose vegetative activity seems to occur only when favorable conditions allow

spores to germinate and to produce mycelia. Perhaps we should call such sporogenous organisms (molds, actinomycetes, as well as spore-forming bacteria) "semi-zymogenous".

It is interesting in this connection to remark that Winogradsky was undoubtedly looking, in part, at Actinomyces spores in his soil preparations when he thought he was studying the indigenous flora. He remarks (50) that the bacteria of the zymogenous flora are primarily large rods, those of the "autochthonous" flora cocci. Now the writer has examined soils from all over the United States, and has never found true micrococci in any abundance; it seems hard to believe that the soils of France can be as different as Winogradsky's statement seems to indicate. The only coccoid organisms observed by the writer have been the very abundant Actinomyces spores and the less common coccoid forms (arthrospores?) of the group recently designated (15) *Arthrobacter* (called *Corynebacterium* by Jensen, 24). Accordingly it is not impossible that a large part of the "autochthonous" forms seen by Winogradsky under the microscope were actually Actinomyces spores.

Species identification among this group has proved difficult. More will be said about this in the later section of this paper dealing with methods of identification of common soil bacteria.

(*c*). *Spore-forming bacteria.* Most prominent among the spore-forming bacteria in soil are the strongly proteolytic species, *Bacillus cereus*, *B. mycoides*, *B. megatherium*, and one or two others. These forms are the most common rapid gelatin-liquefiers of soil origin and have been found by the writer (5, 6) to comprise about 10% of the colonies that develop on gelatin plates inoculated with soil. Other non-proteolytic or less strongly proteolytic spore-formers exist in soil, but are infrequently found on ordinary plates. To demonstrate their presence it is necessary to heat the soil (or soil infusion), before plating, to a temperature sufficient to kill vegetative rods. These last mentioned types are therefore rarely encountered by the student of general bacteriology.

Even before Winogradsky proposed the term "zymogenous" and pointed out the significance of that part of the soil flora, it was realized what must be the function of these spore-formers. It was pointed out (5) that the great constancy of bacterial spores, with but rare occurrence of vegetative forms, must mean that these very active proteolytic species remain normally in soil in inactive form, germinating and multiplying for brief periods only when supplied with proper nutrients. In fact, it was apparently the present writer's conception of the activity of spore-formers which suggested to Winogradsky that other organisms might act similarly and hence comprise a zymogenous flora. The conception is now generally accepted as correct.

Spore-formers have been studied from the taxonomic angle more thoroughly than any other soil forms. There is still some dispute as to where to draw the lines between species in this group; nevertheless, it is true that species identification is easier among them than in the case of any other group of soil bacteria. Methods for the identification of the most common species are given below (p. 267).

(*d*). *Non-spore-forming bacteria.* Apparently the bulk of soil bacteria (at least so far as concerns the flora developing on ordinary plates) do not belong among the zymogenous types. Almost the only non-spore-formers of the zymogenous type are the fluorescent pseudomonads. They may constitute a single species (*Pseudomonas fluorescens*) or a group of closely related species. The writer prefers to regard the forms as comprising a single species, varying enough in its chromogenesis and other biochemical features so that individual strains are often described as separate species. It is one of the most strongly proteolytic types in soil, liquefying gelatin so rapidly at temperatures of 20 to 22 C that one colony may well liquefy a whole plate of gelatin unless the temperature is kept at least as low as 18 C. Unlike *Bacillus cereus* and related spore-formers, *Pseudomonas fluorescens* can fluctuate very greatly in plate count, low on one day, high on the next, and absent entirely on the third day. This fluctuation, naturally, is due to its lack of spores or other resting stage. When its special nutrients are lacking, it must decrease rapidly to a mere minimum, and its numbers become too small to show on plates as highly diluted as must be employed to prevent overcrowding by other forms.

Pseudomonas fluorescens is quite easily distinguished on gelatin plates, although one must not look for its typical fluorescence under such circumstances. The identifying features will be discussed later.

The Indigenous Flora

In contrast to the zymogenous flora, the indigenous types are those that maintain fairly high and quite constant numbers, without showing appreciable increases or decreases according to presence or absence of special nutrients. Their exact function in soil is not fully understood; in fact they have often been neglected by students of soil bacteria. The present writer has always given much attention to them, chiefly because of the interesting speculation as to what rôle might be played by such numerous but unspectacular organisms. Although this interest began many years ago, it must be confessed that assigning a rôle to them is still largely speculative. Doing so is difficult for two reasons: their constant numbers make it impossible to correlate numerical fluctuations with definite activities in the soil; and when isolated and studied in pure culture, they prove to have so few positive biochemical characteristics that it is hard to assign any known chemical transformation to them. They seem to utilize practically the same nutrients as higher plants, e.g., nitrates and ammonium salts, and probably they maintain a low level of activity in soil, utilizing soluble forms of nitrogen as they are produced by the ammonifiers and nitrifiers. Conceivably therefore they may serve as rivals to higher plants, since they draw on the same sources of nitrogen; their rivalry cannot be serious, however, as bacteria are short lived, and are readily decomposed on death of the cells. It is even possible that their presence may be useful to plants in preventing the leaching out of soluble nitrogen when there are no plants to utilize it.

Their lack of strong fermentative reactions makes it difficult to classify them because they are so much alike physiologically. Furthermore, considering that,

as Lochhead and Taylor (29, 30) remark, they are "physiologically unstable", one has a problem of classification about as difficult as presented anywhere among bacteria. Almost the only progress that has been made in the way of classification has been on the basis of morphological features. On the basis of morphology we may consider four groups as follows.

Actinomycetes. As stated above, true actinomycetes (of the genus *Streptomyces*) may or may not be indigenous, and the writer prefers to regard them as probably zymogenous. Possibly some species may be zymogenous, the others indigenous; but since species differentiation is still too difficult to provide much idea as to the relative abundance of the different species under varying conditions, we cannot be sure. Their spores are almost universally present in large numbers, but there is good reason to believe that they become active only on special occasions.

Arthrobacter species. There are large numbers of forms present in soil which are intermediate between true bacteria and true actinomycetes; they are somewhat pleomorphic, showing rod-shaped, coccoid, and mycelioid forms. Jensen (24) called them species of *Corynebacterium*, Krassilnikov (26) of *Mycobacterium*. The writer has not accepted either of these proposals to place them in genera which are typically pathogenic, and originally named the most prominent species (in the soils studied) *Bacterium globiforme*. More recently in collaboration with Miss Dimmick (15) the proposal has been made to amend Fischer's name *Arthrobacter* (originally a *nomen nudum*, i.e., a genus without any species) to include these forms. These *Arthrobacter* forms resemble actinomycetes only in their occasional production of short mycelia; in type of growth they are like ordinary bacteria. They produce very small (punctiform) colonies on agar or gelatin media, and can be distinguished from the following organisms only by isolation and study to determine whether the typical morphological changes can be observed.

Non-pleomorphic non-spore-forming rods (Agrobacterium). There is a group of non-spore-forming rods in soil, essentially like *Arthrobacter* in physiology, but differing from it in showing no tendency toward branching or coccoid forms. They were originally thought to be close to *Alcaligenes*, because of their failure to produce acid or gas from sugars; but pointing out their difference, the writer (14) subsequently proposed the genus *Agrobacterium* to include them. The type species of this genus is *Agrobacterium tumefaciens*, a plant pathogen; and its best known non-pathogenic species is *Agrobacterium radiobacter* which shows much superficial similarity to the legume nodule bacteria (*Rhizobium*, spp.). Isolation of *Agrobacterium radiobacter* from soil usually requires special media; but there are on almost any plate from soil numerous colonies of bacteria that undoubtedly belong to the group, although they have not been given specific names, because of lack of positive characteristics on which to base specific distinctions. It is still uncertain whether few or many such species exist in the soil.

Micrococci. Mention should be made of this group here although the writer does not regard it as an important part of either the indigenous or zymogenous floras. It must be mentioned because Winogradsky, from microscopic observa-

tion of soil, concluded the "autochthonous" flora to be made up primarily of small cocci. The present writer, however, has found true cocci so rarely in soil as to be very doubtful whether those few that are found on the plates are of actual soil origin. Time and time again, however, cultures have been isolated from soil which seem to be micrococci when first examined; but after continued study it has been learned that their earliest stage is a rod form, and it has been concluded that they are actually *Arthrobacter* forms, the coccoid stage of which bears a striking resemblance to a pure culture of a micrococcus. After having this experience repeatedly, without ever observing a strain which is always coccoid in form, in young as well as in old culture, one naturally acquires considerable skepticism as to the occurrence of true micrococci among the indigenous bacteria of soil. Accordingly this group is mentioned here, merely to dismiss it from further consideration.

Methods of Identification

One of the main objects of this paper is to assist students in soil bacteriology in identifying the members of the general soil flora which they cannot help but encounter if soil is plated on gelatin or agar media. The remaining section of this article deals with that subject. It must be recognized in advance, however, that the flora developing on such plates are only a part of the total soil flora, and may not comprise the most important bacteria. This statement is so true that the majority of soil bacteriologists regard plate counts of soil as of no real significance. Nevertheless, the plating of soil is still an interesting procedure, a preliminary step toward the isolation of pure cultures; and the study of such pure cultures in relation to soil activities may eventually solve soil problems that are still baffling. Accordingly it seems well to summarize the information now at hand which helps in identifying the organisms developing on such plates.

Methods employed. There are numerous media that may be used for plating soil when the object is isolation of members of the general flora; but two conditions must be maintained: little organic matter in the medium, and low temperature incubation. Peptone media are specially unsatisfactory, as peptone permits the overgrowth of spore-formers and proteolytic pseudomonads which prevent colonies of the more common but slowly growing bacteria from developing. The same unsatisfactory result occurs if incubation is carried on at temperatures higher than room temperature. If gelatin media are used, no nutrients should be added other than the salts normally present in tap-water, and even the tap-water may be replaced by distilled water without appreciably lowering the count. If agar is employed, nutrients must, of course, be added; but in addition to mineral salts it is well to include no more than 0.1% of glucose and a similar amount of some amino acid, ammonium salt, or nitrate. Various formulae for such agar media have been proposed, each having its own advocates among soil bacteriologists; apparently about the same flora develops on any of them, and apparently about the same counts are obtained, provided incubation is long enough and at a low enough temperature. Gelatin plates should be incubated at 18 C; agar at not over 25 C. Gelatin plates usually must be studied on about

the fifth to seventh day; agar plates may be incubated 10 to 14 days, and it is often desirable to do so, because of the slow growth of the most numerous soil bacteria.

When the medium is to be employed for the isolation of pure cultures for study, the writer prefers 12% gelatin, in tap or distilled water, pH 7.0. This medium is preferred because of the larger number of bacteria that may be recognized from their colonies. No one denies the obvious disadvantages of gelatin, chiefly arising from the danger of rapid liquefaction destroying a whole plate or at least the greater part of it. This liquefaction, however, is much less in tap-water or distilled water gelatin than in nutrient gelatin, and may be further minimized by incubation at 18 C. This low temperature is much easier to secure under modern methods of temperature control than it was formerly; when it is employed, and six plates (three of about 1/100,000 dilution and three of 1/200,000) are poured, it is rare that there are not enough satisfactory plates for study after five days, and incubation for seven days is often possible. The disadvantage of gelatin is outbalanced, in the writer's opinion, by the large number of types of colonies that can be distinguished in it.

The medium used for isolation may be almost any nutrient agar, as practically all the bacteria developing on any of the above mentioned plating media will grow on it. Isolation presents no difficulties except in the case of the numerous punctiform colonies in gelatin which sometimes fail to grow when fished by the inexperienced laboratory worker; for these organisms a useful technic is to employ a needle with a flattened point, and to pass the point around the colony once before the wire is completely cool, thus melting out a small block of the gelatin which can be lifted out bodily on the flat end of the wire and transferred to the agar slant. Other points of technic to be observed will become evident in the following directions.

Classification of colonies. Seven types of colonies can be recognized on gelatin plates:

A. Large, liquefying, rhizoid to mycelioid. (Typical *B. mycoides* R colonies.)

B. Rather large, liquefying, with a granular pellicle which often shows concentric structure. (Typical *B. cereus* R colonies).

C. Rather small, liquefying, with a white flocculent center surrounded by a clear zone. (Colony of *B. megatherium* and the S forms of various spore-formers.)

D. Small to very large, liquefying, structureless and quite clear, a single colony being capable of liquefying the entire plate if given the opportunity. (Typical *Pseudomonas fluorescens* colonies.)

E. Under 3 mm diameter, non-liquefying, hard consistency, showing filamentous margin under low power of microscope; surrounded by a brown halo. (Certain *Streptomyces* colonies.)

F. Like E but without the brown halo. (Certain *Streptomyces* colonies.)

G. Punctiform, non-liquefying, of soft consistency, with entire margins as shown under low power of microscope. (*Arthrobacter* and *Agrobacterium* colonies.)

Of these seven types of colonies, the last three may include as much as 90% of the colonies on the plates. Types E and F are the actinomycetes (*Streptomyces*) colonies: type G, which cannot be distinguished from F without touching with a needle or examination under a microscope includes the *Arthrobacter* forms and the non-pleomorphic non-spore-formers (*Agrobacterium*) which make up a large percentage of the indigenous flora.

Occasionally other types of colonies may be encountered, conspicuous because of red, yellow, or orange chromogenesis. None of these is common enough to deserve special mention, however, except for an orange liquefying colony (often 10 to 20 mm in diameter) which, although usually absent, may sometimes be one of the most numerous colonies on the plate; the writer has identified it as probably *Pseudomonas caudatus*, originally described by Wright (52) in 1895.

Before going ahead with a key to the identity of organisms developing on such plates, one point must be emphasized. Bacteria growing on gelatin, or such agar media as those mentioned, are so numerous that, to prevent overcrowding of the plates, soil must be diluted 1 to 100,000 or more. It is obvious that bacteria occurring in numbers of 100, 1,000 or even 10,000 per gram are all but excluded from such plates, and if their colonies do appear they cannot be told from chance air contaminants; yet organisms occurring in the order of 1,000 per gram may well have an important rôle in soil activities. They are excluded from the present account not because of failure to realize their presence and possible significance, but because they are not encountered in the ordinary plating technic.

It is theoretically possible to obtain by plating methods any desired organism or group of organisms occurring in these smaller numbers in soil, by devising a special medium adapted to the bacteria in question but preventing the growth of the more abundant forms. For example, certain special plating media for *Agrobacterium radiobacter* have been devised (21, 36, 38) by which it is possible to secure this organism directly from soil, at dilutions of around 1/10,000. This organism also grows on ordinary media and presumably is responsible for an occasional punctiform colony appearing on them, but is easily overlooked; special media have been devised for it only because it is a particularly interesting species due to its close relation to the legume nodule organisms. Obviously, there must be many soil bacteria of similar frequency that have never attracted sufficient interest to have special media devised for them. All such organisms have to be omitted from the present survey of the field.

Key to predominant groups and species

Gelatin colonies rapidly liquefying

Colonies rhizoid or filamentous to naked eye.....R forms of *Bacillus mycoides*.

Colonies with granular pellicle which is often concentrically ringed.....R forms of *Bacillus cereus*.

Colonies with a small floc of white granules at center. May be *B. megatherium*, or the S forms of *B. cereus* or *B. mycoides*. To distinguish between these three, inoculate into standard peptone agar slants.

Growth smooth, soft, with a tendency to become a dirty pink. Rods usually over 1 μ in diameter; spores about 1.2–1.5 μ; chains of spores or sporangia rarely observed.....*Bacillus megatherium*.

Growth colorless, smooth, soft, if remaining in the S phase; but wrinkled, membranous if reversion to R form has taken place (as often happens). Rods usually 0.6–0.8 μ in diameter; sporangia swollen and usually remaining in chains for some time; spores about 0.8–1.0 μ ..... *B. cereus.*

Growth same, if remaining in S phase, but rhizoid if reversion to R form has occurred. Morphology exactly like *B. cereus* ..... *B. mycoides.*

Note: Smith (39) insists that *B. mycoides* is only a variety of *B. cereus*. It is certainly true that their S phases are indistinguishable.

Colonies very large, if full growth has taken place, smooth and structureless, with only a minimum of cloudiness ..... *Pseudomonas fluorescens*

Note: Fluorescence can usually be demonstrated by transferring to agar slants, especially if nitrate is present. Absence of fluorescence, however, is not a character of diagnostic importance.

Gelatin colonies small, with little or no liquefaction

Colonies varying in size from punctiform to about 3 mm, hard to the touch, with filamentous margins, as shown under low power of microscope; often surrounded by brown halo. (The larger colonies all easily recognized, but those too small to permit demonstration of the typical tough consistency require careful microscopic examination to be sure they belong here.) ..... *Streptomyces* spp.

Note: There may possibly be more recognizable species in this genus than in any other group of soil bacteria; yet species identification is difficult, and at present is attempted only by specialists of the group. Distinctions between species are based on: (*a*) certain morphological features which are difficult to describe precisely; (*b*) chromogenesis. Chromogenesis is the most striking feature, and is frequently of real diagnostic value; but it must be used with caution, because the pigments produced are ordinarily pH indicators, and to use then for species distinction one must either control the final pH, or at least take pH into account. Because of these difficulties no key to the species of this genus is given here, although there are 73 of them listed in the Sixth Edition of Bergey's Manual.

Colonies usually punctiform, practically never over 2 mm, soft, and with entire margins, as shown by low power of the microscope. These comprise partly simple non-spore-forming rods (*Agrobacterium*) and partly *Arthrobacter* types. To distinguish between them, slant cultures should be made on standard agar, and daily microscopic preparations made for 4 or 5 days. From the appearance of these the following two groups can be recognized:

Remaining continuously in rod form or sometimes oval in shape *Agrobacterium* spp.

Note: The few definitely named species in this genus, *A. radiobacter* and certain plant pathogens, are not sufficiently abundant in soil to appear on ordinary plates. There are always, however, numerous *Agrobacterium* colonies of unnamed species; perhaps one, or two, or many species are represented.

Appearing as rods for 12 to 48 hours and then becoming spherical; large spherical bodies (termed cystites by Jensen) are found, and branching forms occur in liquid media. These *Arthrobacter* species may be distinguished from one another by the certain morphological features and by the presence or absence of yellow chromogenesis. The two species which the writer has found among the predominant soil forms are both non-chromogenic and are so regular in morphology after 3 or 4 days on ordinary agar as to appear like micrococci; whereas another well-known but apparently less common form, *A. helvolum*, is yellow and shows considerable morphological variation in such cultures. The two found commonly in soil by the writer may be distinguished as follows:

With diastatic action on starch, as shown by starch agar plates
Arthrobacter globiforme.
Showing no diastatic action on starch agar plates.....*Arthrobacter simplum*

This key is very crude and perhaps over-simplified. It is not intended to permit the identification of every organism that may be found on plates from soil; in fact, it is not intended as a complete key to species of even the predominant types (as evident from the above "note" under *Streptomyces*). It is offered chiefly in order that a beginner in soil bacteriology may employ it to find his way into the field and to get some idea of the identity of the forms he is most likely to encounter.

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